THE ADAPTATION OF TETRAHYMENA TO A HIGH NaCl ENVIRONMENT[1]

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Many groups of animals have successfully adapted to an osmotically altered environment or osmotic stress. Response and adaptation to a different osmotic and ionic environment have enabled representatives of all major phyla except the echinoderms to invade fresh water from the sea. Other examples are invasion of the sea by fresh-water teleosts, adoption of fresh water for larval development by terrestrial insects, and return to fresh water by terrestrial pulmonate snails. Similar osmotic and ionic problems are faced by estuarine animals and by animals migrating from fresh water to the sea (or *vice versa*) to breed. The problem in these latter instances is met anew by single individuals, as well as over many generations. (For further discussion, see Krogh, 1939.)

The present study was concerned with the adaptation to an osmotic stress by *Tetrahymena pyriformis*, a ciliate which normally lives in fresh water. The stress was introduction into a high NaCl environment. The process of adaptation was investigated from the initiation of the stress through many generations. There appeared to be selection for stress tolerance at the first generation after the stress, and selection for ability to regulate NaCl over many generations. Inorganic ion regulation in the normal and adapted cells was investigated and compared.

Tetrahymena is suited to a study of this nature for several reasons:

(1) It has been known for some time that *Tetrahymena* can adapt to an environment much more concentrated than fresh water (Chatton and Tellier, 1927; Loefer, 1939).

(2) Large homogeneous quantities of cells in suspension needed for physiological studies could be grown easily and quickly.

(3) Studies encompassing many generations are feasible since *Tetrahymena* has a short generation time.

(4) Osmotic regulation by cells is probably effected in large part by controlling particular inorganic ions (*cf.* Brown and Stein, 1960). The main features of inorganic ion regulation in normal (unadapted) *Tetrahymena* are known (Dunham and Child, 1961). *Tetrahymena*, like other fresh-water protozoa and cells of lower fresh-water invertebrates, maintains itself hyper-osmotic to its environment, and also maintains remarkably constant potassium and sodium concentrations over a wide range of hypo-osmotic environmental concentrations. *Tetrahymena* maintains in normal environments a higher potassium concentration than sodium or

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chloride, sodium being more concentrated than in the environment in very dilute environments. *Tetrahymena* extrudes sodium into more concentrated environments, but the extrusion is maximal at a relatively low external sodium concentration (20 m*M*). Potassium is accumulated actively and independently of sodium extrusion. *Tetrahymena* has limited ability to regulate its ion concentration in hyperosmotic environments. These findings have been corroborated by Andrus and Giese (1963).

There is no general agreement on the use of terms pertaining to adaptation and related phenomena. For the purposes of this study, the following terms and definitions are used. The first response of an individual organism to an environmental stress is called the *immediate response* (*cf.* Prosser, 1958). The individual is not changed in any way that is not readily reversed by removing the stress.

The second response, occurring after a longer exposure of an animal to stress, is called *acclimation* (*cf.* Prosser, 1958). This involves compensation in which case the individual is changed in ways that would not readily revert to the original condition upon removal of the stress. As Prosser (1958, p. 168) put it, there is "a new equilibrium of rate functions." Immediate response and acclimation do not involve changes in the genetic constitutions of the individual and therefore are not inherited (though they are certainly a reflection of the genetic makeup of the individual).

A population which has survived an environmental stress for at least one generation may be different from the population of the previous generation, the changes being associated with changes in the gene pool of the population. The stress in this case is *selection pressure*, which may be defined as all causes of systematic and heritable change in populations between one generation and the next that do not directly involve mutation or introduction (or loss) of genetic material from outside (or out of) the population (*cf.* Simpson, 1953, p. 138, and Lerner, 1958, p. 5).

Adaptation is the acquisition within a population of heritable advantageous (adaptive) characteristics, resulting from selection, mutation, introduction into the population, etc. Adaptation may suit a population to an altered environment, or improve the relation of a population to a constant environment.

Adaptation resulting from selection of initially more tolerant individuals is called *preadaptation*. Preadaptation has been defined as the "chance adaptive effects of variation" in a population (Emerson, 1949, p. 642).

Heritable variability exists in every natural population, including those comprised of asexually produced descendants from one ancestor, according to Simpson (1953, pp. 61 and 65).

Adaptation or acclimation to osmotic and ionic stress under quantitatively defined conditions has been reported for a number of animals. Important examples are *Cordylophora* (Kinne, 1958), mosquito larvae (Wigglesworth, 1938), grapsoid crabs (Gross, 1961), the crab-eating frog (Gordon *et al.*, 1961), the eel (Keys, 1933; Krogh, 1939), a number of ciliates (Gause, 1941 and 1942; Loefer, 1939; see Loefer for earlier references), and *Amoeba mira* (Mast and Hopkins, 1941). For additional examples and references, see Prosser (1955) and Bullock (1960).

Preliminary reports of the present work have been published (Dunham, 1961 and 1962).

Methods and Materials

Tetrahymena pyriformis strain W, an asexual amicronucleate strain, was cultured axenically in 2% proteose-peptone (normal medium) or in 2% proteose-peptone + 1% NaCl (high NaCl medium). One-liter Roux bottles containing 500 ml. of medium were inoculated with 5 ml. of medium of a culture in the log phase of growth. Cells were harvested by gentle centrifugation after 4–6 days' growth (normal animals) or 9–12 days' growth (adapted animals) at 22–24° C.

Adapted cultures were started by transferring normal animals with a platinum loop directly into the high NaCl medium without culturing through media of intermediate NaCl concentrations. Failure of adapted cultures to appear was rare.

Some aspects of the process of adaptation were investigated by observing single cells introduced into drops of media of several NaCl concentrations at 23° C. Parameters observed were immediate osmotic response, subsequent recovery, and cell division. NaCl concentrations in the media were 35 m*M*, 120 m*M*, or 200 m*M*. The media also contained 5 m*M* KCl, 0.5 m*M* $MgCl_2$ (K and Mg are growth requirements of *Tetrahymena;* Kidder *et al.*, 1951), and 0.1% proteose-peptone. Drops about 5 mm. in diameter could be kept under mineral oil indefinitely without evaporation. Clones developed and remained "healthy" for as long as a month under these conditions. No special measures were taken to keep the clones axenic.

Methods for packed cell volumes, dry weights of packed cells, number of cells per unit volume, preparation of cell extracts, and analyses of K, Na, and Cl concentrations in cell extracts and media, were as previously reported (Dunham and Child, 1961). Total exchangeability and kinetics of exchange of intracellular Na were determined using Na^{24}. The isotope was obtained from Oak Ridge National Laboratories as NaCl in HCl solution, and was neutralized with NaOH before use. Trace amounts of Na^{24} were added to cell suspensions, samples were removed periodically, and the cells were spun down. The experiments were never complicated by concomitant net fluxes of Na. Counts per minute of wet samples of medium and cell extracts were determined with a Geiger-Muller detector of a NaI-Tl crystal scintillation well detector. All counts were greater than six times background. Per cent exchanges of intracellular Na were calculated from the specific activities of the medium and of the cells, after appropriate corrections for extracellular space.

Extracellular spaces of packed cells were determined using C^{14}-inulin, added to cell suspensions within 30 seconds prior to centrifugation. Radioactivity of dried samples of the supernatant and of cell extracts was determined in a gas flow, windowless counter. Total inulin concentration was always less than 0.05%, which is negligible osmotically.

Results

Process of adaptation

The method was described above for studying the process of adaptation by observing immediate response and clone formation of cells in drops of medium under mineral oil. The results of these experiments are collected in Table I. The controls for this series of experiments were performed as follows: (A) Single normal cells were placed in drops of 35 m*M* NaCl medium, and (B) single adapted

cells were placed in drops of 200 m*M* NaCl medium. All cells survived to divide, showing that failure to do so cannot be attributed to handling of the cells, or to deficiencies in the media. In all experiments described in this section, a successful clone (taken as at least 32 animals, the product of 5 or more generations) never failed to develop if a cell survived to divide once. Therefore the number of surviving cells was always the same as the number of successful clones formed.

(C) Normal cells were introduced singly into drops of 200 m*M* medium. Within seconds all cells became flattened, due to osmotic loss of water. After 30 minutes, all cells had swelled somewhat. Most cells were nearly immotile. After three hours, only the few cells which eventually survived to divide were still motile. Division occurred from 10 to 30 hours after introduction into the 200 m*M* medium. (Normal generation time was 3–4 hours; see below.) No mortality was observed in the subsequent several generations. After the first division, the cells were smaller and more nearly spherical than the original cells.

TABLE I

Survival of single normal and adapted Tetrahymena introduced into drops of 35 mM or 200 mM NaCl medium under mineral oil. Results are expressed as per cent survival, i.e., per cent of cells which survived to divide. Clones developed from all cells which survived to divide once

Experiment		Number cells introduced	Number cells surviving	Per cent survival
(A) Normal cells into 35 m*M* NaCl medium.		36	36	100
(B) Adapted cells into 200 m*M* NaCl medium.		70	70	100
(C) Normal cells into 200 m*M* NaCl medium.		702	16	2.3
(D) Normal cells equilibrated in 120 m*M* NaCl medium, then into 200 m*M* NaCl medium.		36	26	72
(E) Cells from (C) and (D), after 8 generations, into 35 m*M* medium.	C)	99	98	99
	D)	35	35	100
(F) Cells from (C) and (D), after 8 generations, into 35 m*M* NaCl medium for 2 hours, then returned to 200 m*M* NaCl medium.	C)	35	33	94
	D)	36	13	36

(D) Normal cells were equilibrated in 120 m*M* medium for 30–45 minutes. No mortality was observed. (No attempt was made to obtain quantitative data on this point.) These cells were then transferred singly to drops of 200 m*M* medium.

(E) Cells from clones from experiments (C) and (D), after 8 generations in 200 m*M* NaCl medium, were introduced into 35 m*M* medium. No significant mortality was observed. This experiment served as a control for experiment (F).

(F) Cells from clones from experiments (C) and (D), after 8 generations in the 200 m*M* medium, were introduced in 35 m*M* medium, allowed to equilibrate for two hours, then returned to the 200 m*M* medium.

Cells from (C) are descendants of cells which survived the stress of direct transfer before acclimating to the high NaCl medium. Cells from (D) are descendants of cells which acclimated to high NaCl medium, but most likely would not have survived the stress of direct transfer, since experiment (C) showed that only 2% of the population were tolerant of the stress. Experiment (F) shows that many

more descendants of survivors from (C) were tolerant of stress than were descendants of cells from (D). These results suggest a heritable difference between normal and adapted cells.

General morphological and physiological characteristics

Average cell volume for the adapted culture was 9.5 $\mu\mu$l. ± 0.47 (S.E., 5 determinations; determined from packed cell volumes, cell counts, and extracellular spaces). Cell volume did not change during the 22 months the adapted culture was investigated. The average cell volume of normal animals was previously reported as 1.83×10^{-5} μl., or 18.3 $\mu\mu$l. (Dunham and Child, 1961). A more recent determination gave 16.0 $\mu\mu$l., and this value will be used in the present report. (Average cell volumes for *T. pyriformis* strain GL reported by various authors, cited by Zeuthen, 1963, ranged from 16 to 25 $\mu\mu$l.) Adapted cells were not only 40% smaller than normal cells, they also had a different shape, being more nearly spherical than the normal cells. This difference is indicated by width:length ratios of cells of the two types measured on phase micrographs of cells from early stationary phase cultures. The width:length ratios were about 0.5 for normal cells and 0.75 for adapted cells.

The per cent dry weight of adapted cells was 28.8% ± 0.53 (S.E., 5 determinations). The per cent dry weight of normal cells was previously found to be 19.4% (Dunham and Child, 1961). This value has recently been confirmed (19.3%).

A reliable difference between the densities of cells of the two cultures was not distinguished with the methods used. It might be expected on the basis of the difference in per cent dry weights that the adapted cells would have a density 3–4% higher than that of the normal cells. However, assuming the densities of the two cell types were both in the range generally observed (1.05–1.10 g./ml.), the average dry weight per cell was calculated to be 3.2–3.4 mμg. per normal cell and 2.9–3.0 mμg. per adapted cell. Therefore, even though the per cent dry weight of adapted cells is 33% greater than that of normal cells, the amount of non-volatile material per cell is very nearly the same. These values for dry weight per cell are similar to those reported elsewhere. Scherbaum (1957) found 4.1 mμg. for *T. pyriformis* strain GL in "normal mass culture." Hamburger and Zeuthen (1960) found 2–4 mμg. dry weight per cell for strain GL, depending upon the phase of growth of the culture.

In order to determine generation times, numbers of divisions in 36 hours were counted in at least 30 clones in drops under oil. Generation time for adapted cells was about 11 hours at 23° C. Cells adapted for only a few generations and for 22 months (about 1500 generations) had nearly the same generation times. For normal cells the generation time was 3–4 hours at 23° C. (Scherbaum and Zeuthen, 1955, reported 2.3 hours generation time for *T. pyriformis* strain GL in 2% proteose-peptone at 28.5° C., the optimum temperature for growth.)

The adapted animals had a much lower motility than the normal animals. However, if a suspension of adapted animals in high NaCl medium was diluted with a sucrose solution isosmotic with the high NaCl medium, the motility of the cells increased immediately, indicating that the high NaCl medium imposes an ionic as well as an osmotic stress.

TABLE II

Ion concentrations, in mM/l., of normal medium and high NaCl medium

	Na	K	Cl
Normal medium	36.5	5	28.7
High NaCl medium	223	5	215

Extracellular space

Per cent extracellular space of packed adapted cells, in media with Na concentrations less than 300 m*M*, was 13.3% ± 0.5 (S.E., 34 determinations). The full range was from 9% to 17%. Extracellular space seemed to be minimal (9–11%) in media with Na concentrations of 130–170 m*M*, and slightly higher on either side of this range. In media with Na concentrations greater than 300 m*M*, extracellular was around 20%. The shapes of the cells are different in various osmotic conditions, being swollen in dilute media, and shrunken and wrinkled in more concentrated media. The variations in extracellular space are no doubt due to differences in packing of the variously shaped cells.

The mean per cent extracellular space of packed normal cells in normal medium was 9.8%, and in high NaCl medium, 15%. Use of radioactive iodinated serum albumin (Risa) for determining extracellular space gave higher values than did using C^{14}-inulin. For normal cells in normal medium, it was 15% (Dunham and Child, 1961). Delaying this centrifugation after adding Risa resulted in increased extracellular space. This shows the cells take up the Risa.

When using C^{14}-inulin, values for per cent extracellular space were not increased by delaying centrifugation. Therefore, no error resulted from uptake of inulin. (Kidder and Dewey, 1945, showed that *T. pyriformis* does not utilize inulin.) Adding unlabeled inulin to a concentration 10-fold greater than that of the C^{14}-inulin had no effect on per cent extracellular space. Therefore, no error results from binding of inulin by cell surfaces.

Unless otherwise indicated, all cellular ion concentrations in this paper have been corrected for extracellular space.

Ion regulation

The cellular sodium concentration (Na_i) in normal *Tetrahymena* in normal medium was 12.7 meq./l. of cells. In normal *Tetrahymena* equilibrated (30 min-

TABLE III

Cellular sodium concentrations (Na_i) in adapted Tetrahymena in high NaCl medium. Time periods show months during which analyses were made after introduction into high NaCl medium. Mean, standard error of the mean, and number of determinations are given

Stage	Time period (months)	Nai (meq./l. cells)
I	1–2	42.8 ± 2.02 (5)
II	6–7	35.5 ± 2.92 (9)
III	10–12	27.0 ± 1.22 (5)
IV	18–22	21.1 ± 0.75 (15)

utes) in high NaCl medium, Na_i was about 105 meq./l. cells (Dunham and Child, 1961). Ion concentrations of these media are given in Table II. Cells of the adapted culture were first analyzed for Na_i two weeks after introduction into the high NaCl medium, and periodically over the subsequent 22 months. Mean values, grouped in four time periods, are shown in Table III. These data show that after two weeks of adaptation, Na_i had been lowered to 60% less than Na_i in normal cells equilibrated a short time in high NaCl medium. Na_i continued to decrease to 80% less than the initial high level.

Adapted cells were equilibrated 30–60 minutes in various dilutions of high NaCl medium, and in media with increased NaCl concentrations. These experiments were performed on cells between stages I and II and in stage IV of adapta-

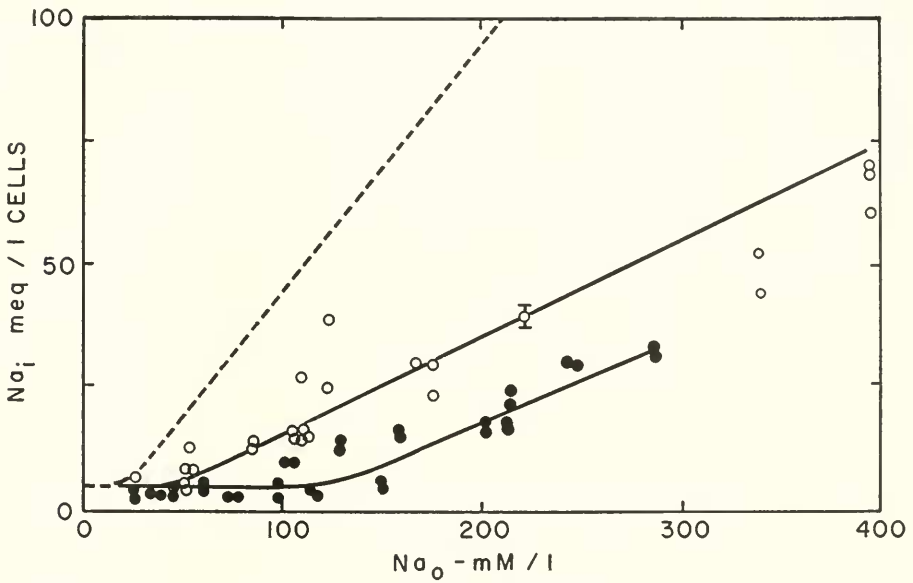

FIGURE 1. Na_i (in meq./l. cells) in normal *Tetrahymena* (dotted curve) and in adapted *Tetrahymena* in stage I–II of adaptation (open circles) and in stage IV (solid circles). Brackets around point for stage I–II cells at Na_o of 227 m*M* indicate standard error of a mean for 12 determinations. All other points represent single determinations after equilibration in media of various Na_o. Curves were fitted by eye.

tion (see Table III). Na_i in adapted cells in high NaCl medium between stages I and II was 39.4 meq./l. cells ± 1.9 (S.E., 12 determinations). Na_i from these experiments is plotted against Na_o (external sodium concentrations) in Figure 1. Included in this figure are comparable data (dotted curve) for normal *Tetrahymena* (from Dunham and Child, 1961).

Na_i in all three kinds of cells in Figure 1 was constant at 3–5 meq./l. in the lower ranges of Na_o. In normal cells Na_i was constant in external Na concentrations up to about 20 m*M*; in stage I–II adapted cells up to 45 m*M*; and up to about 125 m*M* in stage IV cells.

Above these levels of Na_o, Na_i increased roughly linearly with Na_o in all three cell types. The approximate slopes of these linearly increasing portions of the curves were: normal, 0.5; stage I–II, 0.2; stage IV, 0.2.

K_i in adapted cells did not change significantly during the 22 months the culture was studied. Mean K_i was 33.3 meq./l. cells ± 0.7 (S.E., 62 determinations). K_i in normal cells was 31.7 meq./l. cells (Dunham and Child, 1961). Calculated in terms of amount of K per unit number of cells, K_i in adapted cells was 31.6 μeq./10^8 cells and 50.7 μeq./10^8 cells in the normal culture. Whereas the amounts of K per cell are very different, the concentrations per cell volume are very nearly the same in the two cultures.

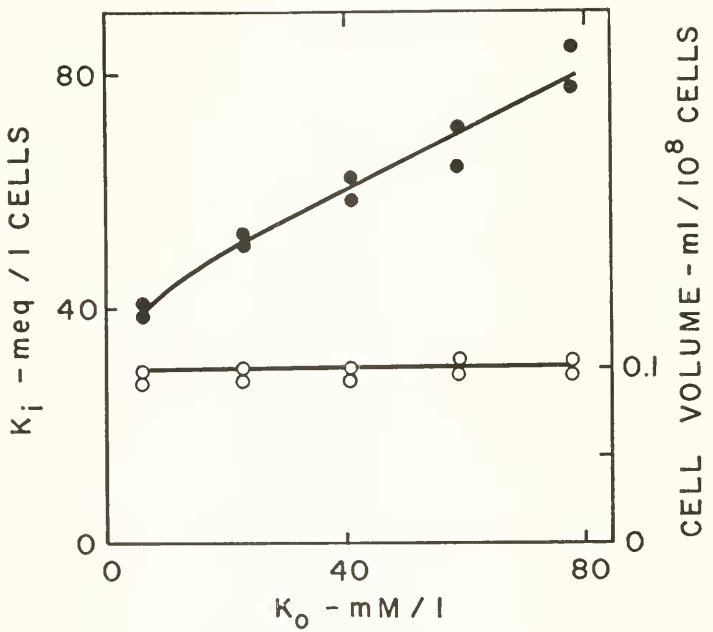

FIGURE 2. K_i in adapted *Tetrahymena* equilibrated 90 minutes in high NaCl media with various increased K_o concentrations. K (solid circles) in meq./l. cells; cell volume (open circles) in ml./10^8 cells.

K_i in adapted cells readily increased with increased K_o. Figure 2 shows K_i in adapted cells equilibrated for 90 minutes in media to which various amounts of KCl had been added. The slope of the linearly increasing portion of the curve is 0.49. The slope of the comparable curve for normal animals was 0.52 (Dunham and Child, 1961).

Figure 3 shows values for Cl_i for adapted cells equilibrated in dilutions of high NaCl medium, and in media made more concentrated with respect to Cl by adding NaCl or KCl. These values are not corrected for extracellular space. No adapted cells were analyzed for Cl_i prior to stage III of adaptation (see Table III). There was no significant difference between stage III and stage IV cells. Below Cl_o of

about 125–140 m*M*, the points for Cl_i fall about a line with a slope of 0.1. The extracellular space fractions in this range of medium concentrations were between 0.09 and 0.12. Therefore, the slope of this line is due to extracellular Cl, and intracellular Cl is constant below 125–140 m*M* Cl_o at about 2.5 meq./l. cells.

Above Cl_o of 125–140 m*M*, uncorrected Cl_i increases with Cl_o with a slope greater than can be accounted for by extracellular Cl. There is considerable scatter of the points, but the slope is approximately 0.2, or, corrected for extracellular space, 0.07. The dotted curve in Figure 3 shows Cl_i corrected for extracellular space according to the above considerations. So Cl regulation in adapted cells corresponds qualitatively to Na regulation, but the constant Cl_i level below Cl_o of

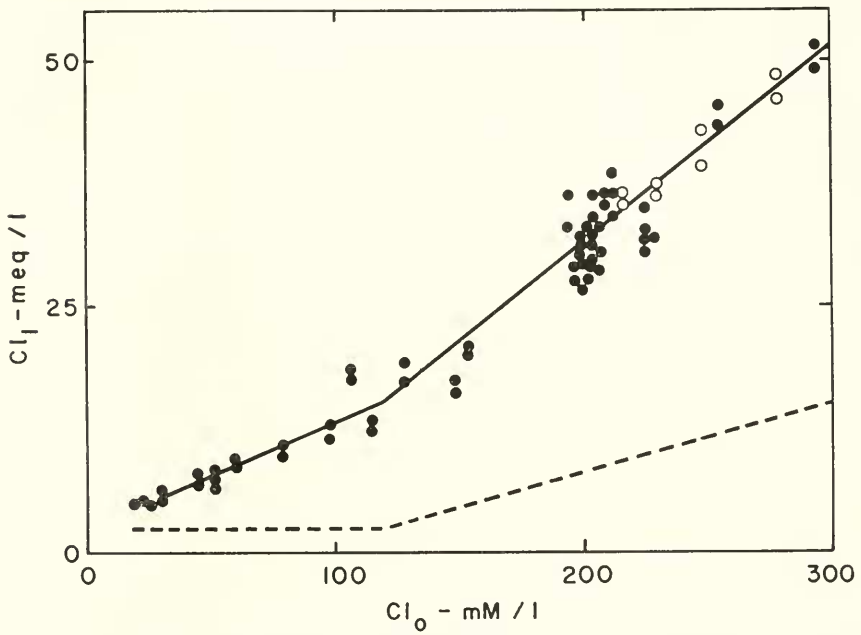

FIGURE 3. Cl_i in adapted *Tetrahymena* in high NaCl medium, equilibrated in dilutions of high NaCl medium, and equilibrated in media with Cl_o increased by adding NaCl (solid circles) or KCl (open circles). Values uncorrected for extracellular space. Curves were fitted by eye. Dotted curve shows data corrected for extracellular space (see text).

125 m*M* is less than the constant Na_i level below Na_o of 125 m*M*, and slope of the increasing portion of the Cl curve is considerably less than in the case of Na. Figure 3 shows that the relationship between Cl_i and Cl_o is the same whether Cl_o is increased by adding NaCl or KCl to the medium.

Figure 4 shows Cl_i in normal cells equilibrated in dilutions of normal medium, and in media made more concentrated with respect to Cl_o by adding NaCl or KCl (Cl_i values uncorrected for extracellular space). The mean of 14 determinations of Cl_i in cells in normal medium was 6.6 meq./l., or corrected for extracellular space, 4.1 meq./l. cells. The constant level in dilute medium is greater in normal

cells than in adapted cells. The region of Cl_o in which Cl_i begins increasing with Cl_o is much lower (40–50 m*M*) in normal cells than in adapted cells, and the slope of the increasing portion of the curve is much greater in normal cells (corrected slope, approximately 0.3).

Kinetics of net changes of intracellular Na and K in normal cells upon changes in external ion concentrations have been reported (Dunham and Child, 1961). Na_i and K_i readily increased upon increase of Na_o and K_o, respectively. Na_i readily decreased, but K_i only very slowly, upon dilution of the medium. Subsequent experiments have shown that Cl_i readily increases or decreases upon appropriate changes of external concentration (unpublished).

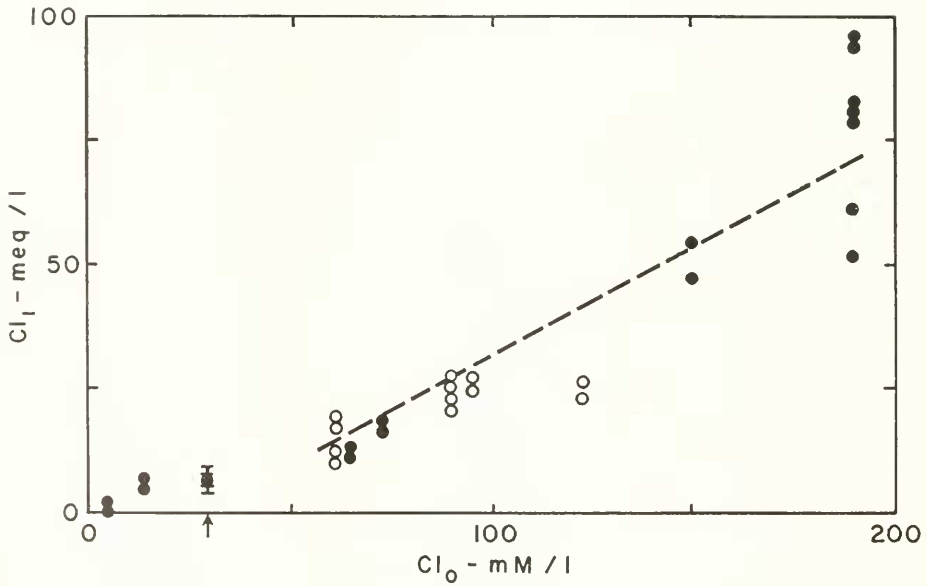

FIGURE 4. Cl_i in normal *Tetrahymena* equilibrated in dilutions of normal medium, in normal medium (arrow on abscissa), and equilibrated in media with Cl_o increased by adding NaCl (solid circles) or KCl (open circles). Values uncorrected for extracellular space. Brackets around point in normal medium show standard error (inner brackets) and standard deviation (outer brackets) for 14 determinations. Curve eye-fitted.

Table IV shows further experiments on rates of changes of cellular ions upon changes of ions in the medium. Experiments A and B in Table IV indicate that adapted cells are freely permeable to Na and Cl.

In experiment A, K_i remained virtually constant. Na_i increased rapidly in the first minute, and then slowly over the next two hours. The cells shrank in the first minute to at most 65% of initial volume, swelled in the next half hour to 78% of initial volume, and remained constant.

In experiment B in Table IV, K_i had decreased somewhat by 50 minutes to 85% of the initial amount of K per cell. Net Na efflux was complete in 15 minutes, whereas Cl_i fell to nearly zero within two minutes.

TABLE IV

Kinetics of net changes of intracellular ions in Tetrahymena upon dilution of or addition of NaCl to the medium

Experiment	Time, minutes	Cell ion content, meq./10^8 cells			Cell volume, ml./10^8 cells
		K	Na	Cl	
(A) Adapted cells. Na_0, initially 225 m*M*, increased to 396 m*M* at zero time.	0	41.7	35.2	—	0.95
	1	38.6	43.2	—	0.62
	27	41.1	44.7	—	0.74
	130	37.1	51.1	—	0.74
(B) Adapted cells. High NaCl medium diluted four-fold at zero time.	0	38.6	36.4	17.3	1.14
	2	38.8	20.2	0	2.00
	15	29.1	6.3	0	1.57
	50	32.5	5.2	0	1.43
(C) Normal cells. Equilibrated 45 minutes in normal medium with NaCl concentration increased by 170 m*M*. At zero time this medium diluted with normal medium to a NaCl concentration of about 62 m*M*.	0	38.9	100	102	0.89
	1.5	41.1	52.2	35.5	1.47
	20	31.6	27.5	9.8	1.23

The kinetics of net influx of Na after Na_0 is increased were of the same general order in normal and adapted cells. Net efflux of Na upon dilution of the medium was slower from adapted cells than from normal cells. However, when normal cells were first equilibrated in high NaCl medium, then net efflux of Na upon dilution of the medium is slower, comparable to the rate of efflux from adapted cells. Experiment (C) in Figure 4 demonstrates this point. These results indicate that permeability to Na is comparable in normal and adapted *Tetrahymena*. These data on fluxes were not sufficient for critical calculations of flux rates.

The exchangeability of Na_i with Na_0 in adapted cells was determined using Na^{24} as a tracer. The data are given in Table V. About 70% of Na_i was available for exchange. The amount of non-exchangeable Na (10–11 meq./l. cells) as determined with the tracer is greater than the constant Na_i component of 3–5 meq./l. cells shown in Figure 1. Apparently some intracellular Na which does

TABLE V

Exchangeability of Na_i with Na^{24} in adapted Tetrahymena. Mean per cent exchange (relative specific activity), standard error of the mean, and number of determinations are given. Experiments carried out on stage II cells in high NaCl medium

Time (minutes)	Per cent exchange
1	18.3 — (2)
13–16	51.3 ± 7.4 (5)
95–115	73.6 ± 3.8 (5)
225–250	69.7 ± 17.9 (5)
350	67.9 — (1)

not readily exchange with Na^{24} is nevertheless mobilized when the cells are placed in very dilute medium. Otherwise, the Na not removed from the cells in dilute medium is also probably non-exchangeable.

Discussion

The results of this study might best be considered within the framework of a "microevolutionary" process (*cf.* Lerner, 1958, p. 4). The results can be organized in terms of several conditions and events of this process.

(1) The immediate response to the stress of transfer to high NaCl was observed both in quantitative experiments and by observing cells under the microscope. There was a volume decrease of at least 50% during the first minute, due to osmotic loss of water. There was then a net influx of Na, and a concomitant reentry of water and volume increase. These are passive changes, and involve no compensation by the animal.

(2) The data indicate preadaptive variability in the original population. Two per cent of the cells were sufficiently tolerant of the stress to survive and divide. Seventy-two per cent of the normal population can survive NaCl if they are first equilibrated in an intermediate NaCl concentration. Therefore, the characteristic which distinguished the 2% from the rest of the population is tolerance of stress, and not ability to acclimate to high NaCl.

The data also suggest that stress tolerance was a heritable characteristic. Almost all (94%) descendants of the cells which originally survived stress of direct transfer could also survive a comparable stress. On the other hand, consider cells living in high NaCl after the stress was minimized by equilibration in an intermediate NaCl concentration. A much lower proportion of the descendants of these cells could survive the stress of transfer to high NaCl. Therefore, it may be concluded that cells tolerant of stress pass this character to their descendants.

(3) The high NaCl medium to which the normal cells are transferred represents a greatly increased selection pressure due to osmotic and ionic stress. The survival and reproduction of a small segment of the population represents selection since the resultant population differed genetically from the original one in that nearly 100%, rather than 2%, of its members carried the character for stress tolerance.

(4) The gradual decrease in cellular Na concentration in the adapted cells constituted further adaptation over a long time. In this way, the population became better suited to a constant environment. Most likely there was variability in ability to regulate salt, and the better regulators had some selective advantage, either lower mortality rate or shorter generation time. These better suited animals became relatively more prevalent through selection, and average cellular Na concentration decreased. Selection for heritable characters is a reasonable explanation for this phenomenon, particularly when it is considered that it took place over the course of about 1500 generations (22 months, 11 hours per generation).

The question might be raised, whether these characters, stress tolerance and salt regulatory ability, are passed from one generation to the next by nuclear or by cytoplasmic inheritance. In an asexual organism, no simple test, short of nuclear transplantation, could give an unequivocal answer to this question. However,

inheritance of some cytoplasmic characters may be just as stable and as significant as nuclear inheritance. According to Simpson (1953, p. 83), "Cytoplasmic inheritance has little effect sharply separable from nuclear inheritance."

(5) There was acclimation (compensatory changes at the level of the individual rather than the population) very soon after introduction into high NaCl. Some changes must have taken place before the first division. First of all, the first division was delayed up to 30 hours. Second, the first division was critical for the formation of a new population. No mortality was observed in the subsequent generations, but there was considerable mortality before the first division. And third, the cells were smaller and more nearly spherical after the first division.

Since the amount of dry material per cell was nearly the same for the normal and adapted cells, the smaller volume of the adapted cells is due to a difference in amount of water per cell. So the adapted cells are not simply a smaller, stunted version of the normal cells, but are of altered composition. From one cell division to the next there is the same amount of organic synthesis in the two cell types. The longer generation time of adapted cells might reflect a diversion of energy from growth processes to ion regulation. Toxic effects of the higher salt concentration on metabolism are another possibility.

The simple morphological differences between the two strains (difference in size, shape, and composition) probably are functionally related to the adaptation. Similar changes in other animals have been taken as sufficient criteria for acclimation or adaptation (in *Cordylophora*, by Kinne, 1958; in mosquito larvae, by Wigglesworth, 1938). In the present study, morphological and physiological changes were observed concomitantly. More work is needed to establish the exact relationship between the various types of change. Preliminary studies comparing the electrophoretic patterns of soluble proteins from normal and adapted cells were done in conjunction with the present study. The patterns were identical with the exception of one protein which was in adapted cells in amounts an order of magnitude greater than in normal cells. (For techniques employed, see Crockett, Dunham and Rasmussen, 1964.)

The primary physiological change arising in the course of adaptation was increased capacity to maintain low cellular concentrations of Na and Cl.

Both normal and adapted *Tetrahymena* were permeable to Na and Cl since net fluxes of these ions in or out of cells were observed after changing the external concentrations of Na and Cl. Also, most intracellular Na readily exchanged with Na^{24}.

Active Na transport has been demonstrated in a variety of tissues in a number of animals, both as extrusion from cells, in such systems as human erythrocytes, frog muscle, and squid nerve, and as transcellular transport, in rabbit kidney, toad urinary bladder, and frog skin (see review by Andersen and Ussing, 1960, for references). Na extrusion is probably characteristic of all animal cells.

Na extrusion by *Tetrahymena* was indicated by the constant maintenance of Na over a range of Na_o up to a level of Na_o considerably greater than Na_i. This region of Na_o in which Na_i begins increasing with increased Na_o represents the lowest level of Na_o at which Na extrusion operates maximally, *i.e.*, the saturation level of the Na extrusion mechanism (Dunham and Child, 1961). Na extrusion in *Tetrahymena* has also been demonstrated by Andrus and Giese (1963). In adapted

Tetrahymena this saturation level gradually increased over 1500 generations, to about 6 times the saturation level in normal cells.

A constant level of Na_i is maintained in normal and adapted cells in media with Na_o below the saturation level of Na extrusion. Presumably this constant Na level is a cellular Na component which remains constant even as total Na_i increases in media with Na_o above the saturation level. The mobile Na_i component (Na_i in addition to the constant component) readily underwent net changes, and was linearly related to Na_o. Therefore, mobile Na_i is probably in equilibrium with Na_o (taking into account the gradient imposed by Na extrusion, which is constant above the saturation level) with a constant mobile Na_i:Na_o ratio. The slopes of the linearly increasing portions of the Na_i/Na_o curves must then be functions of the steady-state between Na_o and mobile Na_i.

This slope is called here "apparent free Na space." In normal cells, the "apparent free spaces" for Na and K were the same, suggesting that the slopes were a measure of the fraction of cell volume available for equilibration with the medium, *i.e.*, the osmotically active fraction of cell volume (Dunham and Child, 1961). However, additional data necessitated rejection of this hypothesis. First, "apparent free Na space" decreased with adaptation, but "apparent free K space" was unchanged. Second, "apparent free Cl space" is less than either Na or K "space" in normal and adapted cells.

Therefore "apparent free spaces" represent physiological characteristics of the cells which are specific for each ion species. In particular, the changes in "apparent free spaces" for Na and Cl with adaptation are compensations directed specifically toward regulation of a low cellular salt concentration. The nature of "apparent free space" is obscure, but for reasons given above, "apparent free spaces" cannot be simply a general osmotic compartment of the cell.

Cl regulation in *Tetrahymena* is qualitatively similar to Na regulation. Cl is maintained lower in the cells than in the environment. Cl_i appears to be constant (at least in adapted cells) in dilute media up to some Cl_o level, above which Cl_i increases linearly with increasing Cl_o. The saturation level of Cl exclusion is higher in adapted than in normal cells. These points suggest the possibility that the same mechanism is responsible for Na and Cl regulation. Na extrusion is not coupled with K accumulation in *Tetrahymena*, so Cl distribution may follow that of Na.

However, Na and Cl distributions are not associated in any simple manner. "Apparent free Cl space" is less than "Na space" in both adapted and normal cells. The rate of net Cl efflux from adapted cells after medium dilution is more rapid than net Na efflux. Cl_i increased whether Cl_o was increased by adding NaCl or KCl to the medium. Inorganic ions in *Tetrahymena* are not distributed according to a simple Donnan equilibrium. A separate mechanism for regulating Cl is possible, but this question and the relationship between Cl and cation distributions await further evidence.

In assessing ion distributions across cell boundaries, electrical potential gradients must be considered. Trans-surface potentials might be responsible for the differences in Na and Cl distributions. Direct measurements on *Tetrahymena* are not available. Trans-surface potentials and effects thereupon of varying the ionic environment have been measured in *Paramecium* (Kamada, 1934; Yamaguchi,

TABLE VI

Cellular potassium concentrations in some lower fresh-water invertebrates, determined by elemental analysis. (See Dunham and Child, 1961, Table I, for values from various sources determined by other methods)

Organism	K_i	Source
Acanthamoeba	27 meq./l. cells	Klein, 1959
Pelomyxa	34.5 meq./l. cell water	Riddle, 1962
Amoeba proteus	34 meq./kg. cells	Raab, unpublished
Tetrahymena (normal)	32 meq./l. cells	Dunham and Child, 1961
Tetrahymena (adapted)	33 meq./l. cells	Dunham, present report
Paramecium	30 meq./l. cells	Akita, 1941
Hydra littoralis	21, 30 meq./kg. wet weight	Steinbach, 1963
Chlorohydra	38, 50 meq./kg. wet weight	Steinbach, 1963
Dugesia	38 meq./kg. wet weight	Steinbach, 1962a,
Tubifex	27 meq./kg. wet weight	Dunham, unpublished
Anodonta (muscle)	10.5 meq./kg. wet weight 24.1 meq./kg. wet weight	Hayes and Pelluet, 1947 Florkin and Duchateau, 1950
Chlamydomonas	20 meq./l. cells	Ronkin and Buretz, 1960
Naegleria	24 meq./kg. cells	Dunham, unpublished

1960) and in an amoeba (Riddle, 1962). Unfortunately, reasonable extrapolations cannot be made from these studies to trans-surface potentials under most of the conditions employed in the present study.

The intracellular concentration of K (in meq./l. cells) is nearly the same in normal and adapted cells. The amount of K per cell is less in adapted than in normal cells (50.7 μeq./10^8 cells in normal, 31.6 μeq./10^8 cell in adapted), whereas after passive changes in cell volume, the amount of K per cell remains constant and the concentration changes, in both normal and adapted cells. Therefore, an alteration in K regulation accompanied adaptation to high NaCl, with the result that K concentration is the same in the two cell types. This observation suggests a minimal cellular K concentration (*cf.* Steinbach, 1962b). It is interesting in this regard to note the similarity of cellular K concentrations in a number of lower fresh-water invertebrates, listed in Table VI. This similarity among animals of some relatively unrelated groups is further indication of a minimal protoplasmic K concentration. Energetically, it is advantageous for cells of these animals to be as nearly isosmotic with the environment as possible (for a theoretical treatment, see Potts, 1954). However, there is a level of potassium below which cells cannot function. The common minimal K concentration to which representatives of

several groups have evolved independently suggests that potassium is an inherent, integral protoplasmic constituent with a definite functional role.

Summary

1. A culture of *Tetrahymena pyriformis* adapted to a high NaCl medium, containing 220 m*M* NaCl, was investigated. The concentration of NaCl in the medium of the normal (unadapted) animals was about 35 m*M*.

2. Upon direct transfer to the high NaCl medium, only 2% of the normal cells were sufficiently tolerant of the stress to survive and divide. Data were presented indicating that stress tolerance is a heritable character, and that this character was selected for upon transfer to the high NaCl medium.

3. The cell volume of the adapted cells was 45% less than that of the normal animals. The adapted cells were more nearly spherical than normal cells. Despite the smaller size of the adapted cells, the amount of non-volatile material per cell was the same in normal and adapted cells.

4. The main feature of the adaptation was a greatly increased ability of adapted cells to maintain a low cellular salt concentration. Sodium concentration in normal cells in normal medium was 13 meq./l. of cells. Sodium concentration in normal cells equilibrated in high NaCl medium was 105 meq./l. of cells. Sodium concentration in adapted animals was 43 meq./l. of cells two weeks after starting the culture, and fell gradually to 21 meq./l. of cells in 1500 generations (22 months). This constituted selection for ability to regulate sodium.

5. Two major differences in sodium regulation between normal and adapted cells were observed. First, the saturation level of the sodium extrusion mechanism, 20 m*M* in normal cells, increased to 120 m*M* in the adapted cells. Secondly, the "apparent free spaces" of both sodium and chloride were lower in adapted than in normal cells. The increased ability to regulate cellular sodium in adapted cells was held not to be due to a decrease in permeability to sodium.

6. From experiments utilizing an isotopic tracer, Na^{24}, 70% of cellular sodium was shown to be readily exchangeable with external sodium.

7. Potassium regulation was altered with adaptation such that potassium concentration per unit volume was the same in normal and adapted cells. The striking similarity of cellular potassium concentrations in *Tetrahymena* and a variety of other lower fresh-water invertebrates was pointed out. These points were discussed with respect to a general minimum protoplasmic potassium concentration.

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